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REVIEW

Neutrophils as Components of Mucosal Homeostasis

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SUMMARY

There is recent appreciation that neutrophils contribute significantly more to innate immunity than just their function in battling infection. It is now clear that neutrophils can influence tissue metabolism, the composition of the microbiome, and communication between cell types through the release of microparticles. This article highlights the role of neutrophils in both homeostasis and pathology in mucosal surfaces.

Inflammatory responses in the intestinal mucosa inevitably result in the recruitment of neutrophils (polymorphonuclear leukocytes [PMNs]). Epithelial cells that line the mucosa play an integral role in the recruitment, maintenance, and clearance of PMNs at sites of inflammation. The consequences of such PMN-epithelial interactions often determine tissue responses and, ultimately, organ function. For this reason, there is significant interest in understanding how PMNs function in the mucosa during inflammation. Recent studies have shown that PMNs play a more significant role in molding of the immune response than previously thought. Here, we review the recent literature regarding the contribution of PMNs to the development and resolution of inflammation, with an emphasis on the role of the tissue microenvironment and pathways for promoting epithelial restitution. These studies highlight the complex nature of inflammatory pathways and provide important insight into the difficulties of treating mucosal inflammation. (*Cell Mol Gastroenterol Hepatol* 2017;4:329–337; <http://dx.doi.org/10.1016/j.jcmgh.2017.07.001>)

Keywords: Epithelium; Microbiota; Inflammation; Hypoxia-Inducible Factor; Metabolism.

A primary function of the intestinal mucosa is to provide a selective barrier to the outside. The potential for infection by pathogenic organisms and the necessity to communicate with commensal microorganisms exist on the same surfaces. In this regard, tissue healing after injury occurs in conjunction with the constant flux of new antigenic material and requires that the mucosal immune system appropriately dampen inflammatory and immunologic reactions to harmless ingested antigens. The overlying epithelium plays an important role in coordinating both inflammation and resolution. The epithelium lies juxtaposed to the mucosal immune system and lines the

entire gastrointestinal (GI) tract. Covering a surface area of approximately 300 m², the human adult intestinal epithelium consists of a monolayer of cells with intercellular tight junctions, a complex 3-dimensional structure, and a thick mucous gel layer that provides a dynamic and regulated barrier to the flux of the luminal contents to the lamina propria.^{1,2} It is widely understood that the GI tract exists in a state of low-grade inflammation. Such a state results from the constant processing of luminal antigenic material and the priming of the mucosal immune system for rapid and effective responses to antigens or microbes that may penetrate the barrier.³

The presence of polymorphonuclear leukocytes (PMNs) at sites of tissue injury and infection has long been recognized as a hallmark of mucosal inflammation.⁴ It increasingly has become appreciated that the presence of PMNs at sites of injury do not necessarily prove causation to tissue damage, and, in fact, a number of studies now suggest that PMNs provide important cues that promote inflammatory resolution and a return to mucosal homeostasis. In this review, we discuss recent literature regarding the role of microbiota in the recruitment of PMNs to the mucosal surface, the critical role of PMNs in oxygen metabolism, and implicating PMNs in promoting homeostasis in the mucosa.

Microbiota Promote PMN Recruitment into the Mucosa

The mammalian GI tract plays host to trillions of bacteria, viruses, and fungi, collectively termed the *microbiota*. A finely balanced mutualism exists within the intestinal mucosa, in that microbes are essential for intestinal health but also can be involved in inflammation and pathologic damage.⁵ Because PMNs provide the first line of defense to infection, PMNs frequently interact with the commensal microbiome. In general, PMNs do not initiate inflammation in these interactions with commensal microbes. In past

Abbreviations used in this paper: ATP, adenosine triphosphatase; CGD, chronic granulomatous disease; DMOG, dimethylallylglycine; GI, gastrointestinal; HIF, hypoxia-inducible factor; IBD, inflammatory bowel disease; ICAM-1, intracellular adhesion molecule-1; IL, interleukin; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PHD, prolyl-hydroxylase; PMN, polymorphonuclear leukocyte; SIRTα, signal-regulatory protein-α.

Most current article

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years it increasingly has been evident that the microbiome significantly impacts the development and activation of the immune system, including PMNs.

Circulating neutrophils increase within days after birth in human neonates. In a study of antibiotic treatment of pregnant mice, neonatal pups born to these mice developed a reduced number and altered composition of microbes as compared with mice not treated with antibiotics.⁶ This microbial shift correlated with reduced numbers of neutrophils, fewer Ly6G+ cells in the bone marrow, and decreased circulating granulocyte colony-stimulating factor. This relative neutropenia was similar in germ-free mice. Prior work had identified interleukin (IL)17 as a key regulator of granulocyte colony-stimulating factor and granulocytosis.^{7,8} Indeed, IL17-producing cells were reduced in these antibiotic-treated and germ-free mice. The IL17 production was not dependent on the adaptive immune system because RAG-deficient mice had persistent IL17 production and neutrophil recruitment. The neutropenia associated with antibiotic therapy in these mice resulted in increased susceptibility to sepsis, arguing for a protective role for microbiota-stimulated PMN production and recruitment. This neutropenia was reversed with reconstitution of normal microbiome after antibiotic therapy.

Beyond bacterial load and composition, specific bacteria have been shown to change PMN populations. Segmented filamentous bacteria, for example, have been shown to induce IL17- and CXCR2- (PMN receptor for IL8) dependent recruitment of neutrophils⁹ (Figure 1). Segmented filamentous bacteria are spore-forming, gram-positive, filamentous bacteria ranging between 1 and 2 μm in diameter and as long as 80 μm in length that colonize the intestine of mice at the time of weaning.¹⁰ Unlike the IL17-dependent PMN recruitment in the prior neonatal study, the IL17 production was dependent on the adaptive immune system because PMN

recruitment was not appreciated in RAG-deficient mice. In neutrophil-depleted mice with filamentous bacterial colonization, bacterial expansion and IL17-producing cells were increased, suggesting a negative feedback of neutrophils on IL17 production. Other studies have had similar findings that germ-free and antibiotic-treated mice have decreased neutrophilic response to peritoneal inflammation.¹¹ Recruitment in this experimental model was MyD88-dependent. Another study identified that reconstitution of PMN number in germ-free mice can be achieved by serum transfer from mice with a conventional microbiome, suggesting that a soluble factor (eg, IL17) is integral to PMN recruitment.¹² These studies contribute to the conflicting data regarding whether IL17 is more pathogenic or protective in the GI tract. These data speak to the importance of IL17 to recruitment of PMNs necessary to fight infections. Other studies point to the pathogenic, proinflammatory role of this cytokine, particularly IL17F, in the GI tract in diseases such as inflammatory bowel disease (IBD).¹³

There are likely to be differences between various mucosal surfaces with regard to the contribution of PMNs to mucosal homeostasis. A comparison of the GI tract and the lung for instance, suggests that the role of PMNs on tissue function may be different. This particular aspect has been shown convincingly in vivo. The depletion of circulating PMNs using anti-Gr1 antibodies resulted in the exacerbation of symptoms in a number of different murine colitis models, strongly implicating PMNs as a central protective factor in ongoing inflammation.¹⁴ By contrast, the depletion of PMNs in acute lung injury models appears to have an anti-inflammatory effect¹⁵ and severe disease has been associated strongly with the presence of PMNs, driving the argument that PMNs play a key role in acute lung injury.¹⁶ It is notable that this idea has been revisited to suggest that PMNs can be eliminated effectively through mucociliary clearance

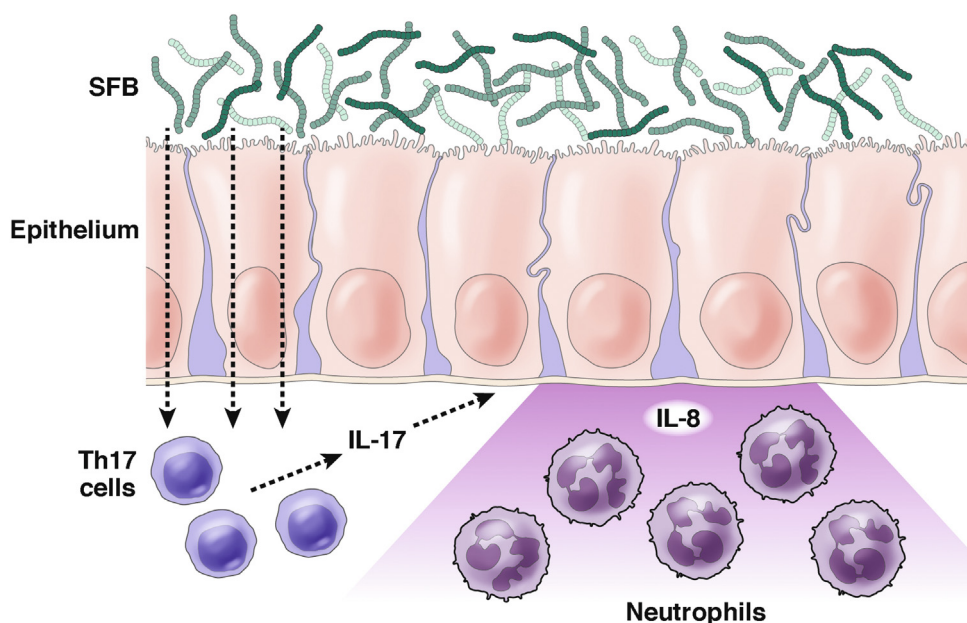


Figure 1. Selective components of the microbiome influence PMN recruitment to the mucosa. The luminal microbiota, including segmented filamentous bacteria (SFB), provide signals (eg, IL23 production from multiple cell types) that drive the accumulation of Th17 cells in the lamina propria. In turn, Th17 cells secrete IL17, which activates the release of chemokines from epithelial cells to promote the accumulation of PMNs at tissue sites.

without damage to the surrounding lung.¹⁷ Nonetheless, these results suggest differences in mechanisms of inflammatory resolution between various mucosal organs.

PMNs and Oxygen Metabolism in the Regulation of Mucosal Function

Once at the tissue site, PMNs fundamentally can change the metabolism of the mucosa. Some recent work, for example, has indicated that PMNs significantly change the availability of oxygen to the surrounding tissue. Compared with other mucosal tissues, the healthy intestine is relatively hypoxic, existing at a P_{O_2} of less than 40 mm Hg.^{18,19} Studies comparing functional responses in epithelial cells from different tissues showed that intestinal epithelia uniquely are resistant to low P_{O_2} environments and that even very low levels of O_2 within the normal mucosa (so-called *physiologic hypoxia*) may represent an adaptation to the steep oxygen gradient that exists across the intestinal lumen.²⁰ These studies lead to the observation that epithelial cells of the colon basally regulate the transcription factor hypoxia-inducible factor (HIF),²¹ one of the global regulators of gene expression in low O_2 conditions.^{22–24} Within the colonic mucosa, it has been shown that the low O_2 conditions that enable microbial short-chain fatty acid production (eg, acetate, propionate, and butyrate) also promote O_2 consumption in a fashion that stabilizes HIF and maintains basal expression of antimicrobial peptides²⁵ and proteins that sustain the mucosal barrier.²⁶

Oxygen utilization via tissue metabolism is exacerbated during inflammation. It recently was shown, for instance, that during acute inflammatory disease, infiltrating PMNs mold the tissue microenvironment in ways that significantly promote the stabilization of HIF.²⁷ An unbiased profiling of epithelial cells after PMN transmigration identified the regulation of a cohort of HIF target genes. By using HIF reporter mice, Gp91^{phox-/-} mice (lack a respiratory burst), and PMN-depletion strategies in acute colitis models, these studies showed that transmigrating neutrophils deplete the surrounding tissue of molecular oxygen in a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase-dependent manner. As a result of the profound oxygen depletion within the tissue microenvironment, transmigrating PMNs transcriptionally imprint a molecular signature onto the surrounding parenchyma (eg, epithelial cells) that significantly reflects the stabilization of HIF. This molecular signature promotes effective HIF-dependent tissue protection. At present, it is unclear whether such imprinting occurs beyond the epithelium and whether such changes are transient or reflect more permanent epigenetic modifications.

Gp91^{phox-/-} mice developed highly accentuated colitis relative to controls. They had exaggerated PMN infiltration, diminished inflammatory hypoxia, and increased microbial invasion.²⁷ A clinical corollary to these findings is that nearly half of patients who lack a functional NADPH oxidase (ie, chronic granulomatous disease [CGD]) present with an IBD-like syndrome.^{28,29} This NADPH oxidase complex is responsible for the generation of reactive oxygen species and is used by PMNs to kill invading pathogens and

commensal bacteria that have breached barriers and interact with NADPH oxidase-expressing cells. Such clinical observations suggest that CGD-associated IBD could represent a failure to resolve acute intestinal insults.

Significant localized oxygen consumption is associated with acute inflammation³⁰ (Figure 2). Numerous studies have shown that such inflammatory hypoxia stabilizes the transcription factor HIF in the surrounding tissue.³¹ Once stabilized, HIF triggers the transcription of a cohort of genes that enable intestinal epithelial cells to promote epithelial restitution.^{20,32–34} Studies have shown that intestinal epithelial cells subjected to low O_2 regulate barrier-related genes in a transcription-dependent manner.³⁵ A number of these target genes subsequently have been validated in animal models of inflammation^{21,36–40} and in human tissues.^{27,41–43} The functional proteins encoded by these HIF target genes include those that localize primarily to the most luminal aspect of polarized epithelia that contribute fundamentally to effective barrier function. These target genes include mucins,⁴⁴ molecules that modify mucins (eg, intestinal trefoil factor),²⁰ xenobiotic clearance,³² antimicrobial peptides,²⁵ and nucleotide metabolism.^{34,35}

The analysis of HIF as a component of the restitution response during mucosal inflammation has guided the development of pharmacologic molecules that function to stabilize HIF and drive the expression of HIF target genes.^{45,46} For the most part, the pharmacologic approach to achieve HIF stabilization in normoxia has involved the inhibition of HIF prolyl-hydroxylases (PHDs), originally discovered as products of genes related to *Caenorhabditis elegans* egl-9,⁴⁷ and subsequently cloned in mammals as PHD1, PHD2, and PHD3. These enzymes were shown to hydroxylate HIF- α in vitro.^{47,48} Targeting the catalytic domain of PHDs initially was achieved by a screen of molecules that interfere with critical cofactors such as 2-oxoglutarate using mimetics that occupy the enzyme (eg, dimethylxalylglycine [DMOG]).⁴⁹ Interestingly, the addition of DMOG to a chemically induced colitis model proved highly effective in promoting the resolution of inflammation.³⁶ A parallel study published at the same time used a different PHD inhibitor (FG-4497) that was based on a screen to identify erythropoietin inducers. Similar to DMOG, FG-4497 blocks the active site of PHDs.⁴⁹ In both studies, HIF-stabilizer treatment was associated with profound pro-resolving functions, particularly related to mucosal barrier function.^{36,39} It is notable PHD inhibitors are currently in development for renal anemia (erythropoietin induction)⁴⁵ and that the use of local oral delivery of an extended-release preparation to the inflamed mucosa could represent a novel therapeutic approach for IBD.

PMNs Monitor Innate Immune Responses at the Surface of the Mucosa

The rearrangements of tight junctions by transmigrating PMNs remain insufficiently understood. Studies using inhibitors of PMN proteases and PMNs from patients with CGD suggests that the mechanism by which PMNs migrate

across tight junctions is not through proteolysis or oxidant production, respectively,^{50,51} but rather requires mechanical impalement of the tight junction.

Studies using functionally inhibitory antibodies have shown that β_2 integrins are required for PMN migration across epithelial surfaces⁵² (Figure 2). β_2 integrins are heterodimeric glycoproteins that exist in 4 forms, each showing a unique α -subunit (CD11a, b, c, or d) and an identical β -subunit (CD18).⁵³ PMN expression of CD11b/18, but not CD11a or CD11c/18, is required for successful PMN transepithelial migration.⁵⁴ The role of adhesion-based receptors, β_2 integrins, are best shown in the genetic disorder leukocyte adhesion deficiency. This is a rare disorder in which patients lack normal expression of the β -subunit CD18 and, as a result, have severe immunodeficient symptoms.⁵⁵ Similar to CGD, leukocyte adhesion deficiency patients often manifest severe mucosal disease.^{56,57} PMNs from leukocyte adhesion deficiency patients fail to migrate across intestinal epithelia,⁵⁴ providing further evidence for the dependence of PMN CD11/18 integrins on transmigration.

Transmigration through the paracellular space is governed primarily by CD47, a membrane glycoprotein of approximately 60 kilodaltons expressed in a polarized

fashion (basolateral) on epithelial cells. CD47 has homology to the immunoglobulin supergene family.⁵⁸ Further studies have shown that CD47 functions in concert with signal-regulatory protein- α (SIRP α), a cell-surface protein containing 3 immunoglobulin superfamily domains and intracellular immunoreceptor tyrosine-based inhibitory motifs.⁵⁹ CD47 is a ligand for Sirp α and studies have shown that CD47 activity is proportional to the expression of Sirp α and that Sirp α -CD47 interactions mediate cell-cell interactions.⁶⁰ Thus, it appears that CD47 is critically important to regulated transepithelial migration (Figure 2).

Although the formation of crypt abscesses may serve as histologic markers of ongoing inflammation, significant evidence suggests that PMN migration into the lumen is an important part of innate immunity. For example, on reaching the apical surface of the epithelium, PMNs bind to intracellular adhesion molecule-1 (ICAM-1), which is expressed in a polarized manner exclusively on the apical epithelial surface.⁶¹ Sumagin et al⁶² recently showed that binding of PMN CD11b/18 to epithelial ICAM-1 is associated with decreased PMN apoptosis, thereby lengthening the life of the PMNs within the lumen of the intestine. These interactions of PMNs with ICAM-1 also were shown to

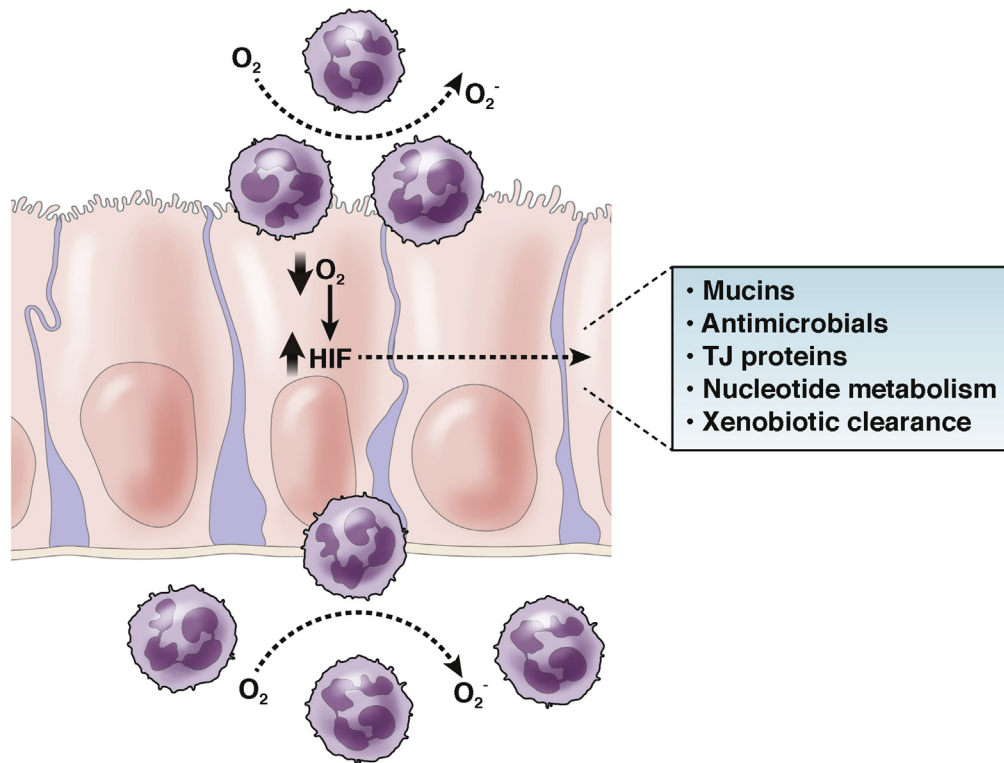


Figure 2. O_2 consumption by transmigrating PMNs stabilizes mucosal HIF and promotes epithelial restitution. (A) Known steps of PMN transmigration. Recruitment signals liberated at inflammatory sites attract PMNs to migrate into and across the mucosa. Initial adhesion of PMNs is mediated by CD11b/18 to a currently unknown basolateral ligand. Movement of PMNs through the paracellular space is mediated by epithelial CD47 and coxsackie adenovirus receptor (CAR) binding to PMN SIRP- α and junctional adhesion molecule-L (JAM-L), respectively. Once at the apical surface, PMNs are retained in a CD11b/18-ICAM-1-dependent manner or cleared through anti-adhesive mechanisms involving epithelial CD55 and PMN CD97. (B) PMN accumulation at such sites become activated to consume large amounts of O_2 via the NADPH oxidase complex. As a result, the local tissue environment becomes deplete of molecular O_2 , which promotes the stabilization of HIF within the epithelium and surrounding parenchyma. The activation of multiple HIF target genes promotes the active resolution of inflammation within the mucosa, particularly related to barrier and antimicrobial function. TJ, tight junction.

activate β -catenin signaling and promote epithelial wound closure and epithelial proliferation. Furthermore, using colon biopsy as a model of wounding, apical ICAM-1 was found to be central to wound closure in the lumen of the colon. Other studies have shown that apically localized CD55 functions as an anti-adhesive molecule promoting the clearance of epithelial-bound PMNs^{63,64} (Figure 2). Surface-expressed CD55 has been shown to interact directly with PMN CD97.⁶⁵

At the luminal surface, PMNs serve as a prominent reservoir for luminal adenosine precursors. Adenosine and its analogs can ameliorate the course of a variety of inflammatory diseases.⁶⁶ After transmigration, PMNs actively release adenine nucleotides, particularly in the form of adenosine triphosphate (ATP) or adenosine diphosphate.⁶⁷ The phosphohydrolysis of ATP and adenosine monophosphate by apically expressed apyrases (eg, CD39) and ecto-5'-nucleotidase (CD73), respectively, represent the major pathways for accumulation of extracellular adenosine.^{34,35,68} Once liberated in the extracellular space, adenosine either is recycled (eg, through dipyridamole-sensitive carriers) or interacts with cell-surface Ado receptors.⁶⁹ Four subtypes of G-protein-coupled Ado receptors exist, designated Adora1, Adora2a, Adora2b, or Adora3, and are classified according to utilization of pertussis toxin-sensitive (A1 and A3) or -insensitive (A2A and A2B) pathways.⁶⁹ Recent work specifically has implicated the epithelial AA2BR in protection afforded by adenosine in mucosal inflammation. For example, Aherne et al⁷⁰ compared Adora2b deletion in vascular endothelial cells [Adora2b[fl/fl] VeCadCre[+]] or intestinal epithelia [Adora2b[fl/fl] VillinCre[+]] and showed a selective role for epithelial Adora2b signaling in attenuating colonic inflammation. Such protection was owing to Ado-dependent increases in adenosine 3',5'-cyclic monophosphate that is accompanied by increased actin polymerization and the cross-linking functions of vasodilator-stimulated phosphoprotein. Given the transient increase in epithelial permeability associated with PMN transmigration, these studies indicate PMN-derived adenosine signaling to the epithelial tight junction provides a signal to close the door after leaving and, as such, serves an important role in mucosal resolution (Figure 2).

Platelets are also a rich source of extracellular ATP. Platelets release nucleotides at high concentrations upon activation by collagen or adenosine diphosphate activation of dense granule release.⁷¹ The co-migration of platelets and PMNs has been observed in intestinal tissue derived from human patients with IBD. Platelet-PMN clusters were found to release large quantities of ATP, which were metabolized to Ado to activate fluid transport into the intestinal lumen. This physiologic response has been suggested to serve as a flushing mechanism for mucosal-associated bacteria as part of the innate immune response.⁷²

PMN Microparticles as Conveyors of Local Information

An area of significant interest is the liberation of microparticles from activated PMNs. Microparticles are

subcellular microvesicles that are shed from membranes under a variety of conditions. Ranging in size from 100 nm to 1 μ mol/L, these membrane vesicles originally were considered cellular debris but recently have become a topic of considerable interest.⁷³ Numerous studies now show that microparticles carry lipid, protein, RNA, and DNA cargo as a means of communication between cells, tissue, and even organs.⁷³ PMN-derived microparticles are increased significantly during inflammation and have shown significant promise as biomarkers, diagnostic tools, and as a contributor to the resolution of inflammation.⁷⁴ For example, Dalli et al⁷⁵ compared the microparticle cargo in microparticles from PMNs in suspension and adherent to endothelial cells. These studies identified more than 400 proteins with nearly half shared between the adherent and nonadherent groups. Most notably, they showed that PMN-derived microparticles were functional and had the capacity to generate leukotriene B4 and to elicit an oxidative burst. This same group has shown that microparticle α -2-macroglobulin promotes inflammatory resolution in murine models of sepsis.⁷⁶ Conversely, other investigators have shown that PMN-derived microparticles can promote inflammation through delivery of myeloperoxidase⁷⁷ and metalloproteinase-9.⁷⁸

Other roles for PMN in Mucosal Immunity

PMNs also play important roles in determining subsequent immune responses in the mucosa. PMNs are a newly appreciated source of multiple chemokines and cytokines because they have been shown to have the capacity to express, produce, and secrete a broad range of both proinflammatory and anti-inflammatory cytokines and chemokines.^{79,80} PMNs also have the capacity to function as suppressor cells. For instance, Bowers et al⁸¹ have shown that when activated appropriately, PMNs can express high levels of programmed death ligand-1, and through this activity can suppress T-cell function. It also is possible that PMNs, through actions on HIF stabilization (see earlier), could be central to regulating the balance between regulator T cells and Th17 differentiation. For instance, Dang et al⁸² showed that HIF-1 transcriptionally activates ROR γ t and attenuates regulator T cell development by binding to Foxp3. Other studies have shown that PMNs recruited to the skin by bacterial infections migrate to the draining lymph node where they augment lymphocyte proliferation.⁸³ This process was shown to be CD11b- and CXCR4-dependent and occurs through direct PMN interactions with lymphatic endothelial cells. Other investigators have shown that CD8 T-cell immune responses to mucosal viral infections are initiated by PMN transport of virus to the bone marrow.⁸⁴ Within these studies, it was suggested that PMNs could contribute to antigen presentation and CD8 T-cell priming.

Conclusions

The trafficking of PMNs to the mucosa represents an important part of the innate immune response. Within the mucosa, restitution of the epithelial barrier defines a critical determinant of a productive inflammatory response. Recent

studies investigating changes within the microenvironment of acute inflammation have shown new important signaling pathways initiated by activated PMNs. Studies in recent years have identified components of the microbiome as central players in productive and pathologic inflammatory responses. Also notable is the shift in tissue oxygenation toward hypoxia, and specifically HIF target pathways associated with barrier restitution and altered cellular bioenergetics that contribute fundamentally to productive innate immunity. These adaptive metabolic pathways activated in response to PMN infiltration represent potentially important therapeutic opportunities for the treatment of mucosal inflammation.

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Conflicts of interest

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